Journal of Reproduction and Development, Vol. 68, No 4, 2022

Opinions and Hypotheses

Genetically engineered animal models for Marfan syndrome: challenges associated with the generation of pig models for diseases caused by haploinsufficiency

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Abstract. Recent developments in reproductive biology have enabled the generation of genetically engineered pigs as models for inherited human diseases. Although a variety of such models for monogenic diseases are currently available, reproduction of human diseases caused by haploinsufficiency remains a major challenge. The present study compares the phenotypes of mouse and pig models of Marfan syndrome (MFS), with a special focus on the expressivity and penetrance of associated symptoms. Furthermore, investigation of the gene regulation mechanisms associated with haploinsufficiency will be of immense utility in developing faithful MFS pig models.
Key words: Disease model pig, *FBN1*, Genetic engineering, Haploinsufficiency, Marfan syndrome

(**J. Reprod. Dev. 68:** 233–237, 2022)

Introduction

The development of genetically engineered (GE) animals has been one of the most significant achievements in the field of reproductive biology in recent decades. GE animals that model monogenic disorders, including cardiac diseases, metabolic and hormonal disorders, and neurodegenerative conditions, play an indispensable role in the study of inherited human diseases [1, 2]. Although most mammalian disease models utilize rodents as the study organism, these animals are genetically, anatomically, and physiologically distinct from humans in several aspects, which limits the extrapolation of experimental observations and results [3]. Pigs and humans share multiple anatomical and physiological characteristics [4]. The recent advent of highly effective genetic engineering technologies, including gene knockout, has led to the development of monogenic disease models based on these animals [5–14]. Multiple studies have demonstrated the successful reproduction of disease pathologies associated with human pathogenic genetic variants after their introduction into the pig genome. Although remarkable success has been achieved in the context of monogenic disorders with clearly dominant alleles, the generation of pig models for diseases caused by haploinsufficient alleles, such as Marfan syndrome (MFS), remains challenging.

The present study compared the characteristics of mouse and pig MFS models, with special emphasis on variable penetrance and expressivity of symptoms, which have been ascribed to haploinsufficiency.

Marfan Syndrome and Animal Models

MFS is an autosomal dominant disease of connective tissue caused by a heterozygous loss-of-function mutation in the fibrillin-1 encoding gene *FBN1*. A reduction in *FBN1* expression results in a decrease in fibrillin-1 deposition, leading to dysregulation of transforming growth factor-beta (TGF-β) and a decrease in the structural integrity of connective tissues due to a lack of microfibrils in elastic tissues [15]. The disease affects several organ systems, including the cardiovascular, skeletal, and ocular systems [16]. This disease primarily manifests as deformities of the cardiac valve apparatus, scoliosis, arachnodactyly, myopia, and lens dislocation [17, 18]. Although significant advances have been made in the diagnosis and treatment of MFS, the premature morbidity associated with this disease is high and warrants rigorous investigation. A better understanding of the factors pertaining to the variable penetrance and expressivity of MFS will aid in the development of advanced treatment modules that will improve quality of life and limit patient mortality.

MFS is characterized by a wide clinical spectrum due to variable penetrance and expressivity in affected individuals [19, 20]. Variations in the manifestation and severity of symptoms, along with differential penetrance, have been reported among affected family members with identical mutations in the responsible gene [21–23]. Furthermore, genotypic-phenotypic associations remain unclear because of marked phenotypic variability among individuals harboring the same pathogenic variant [21]. These findings conclusively demonstrate the pathological variance of MFS, also referred to as reduced penetrance or variable expressivity, among affected individuals. The phenomenon of inter- and intra-familial variability in the onset of MFS can be explained by haploinsufficiency, where a crucial threshold loss of

Received: March 3, 2022
Accepted: May 2, 2022
Advanced Epub: May 23, 2022

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fibrillin-1 function is required for the clinical manifestation of MFS [19]. Consequently, the variability in penetrance and expressivity must be considered when conducting research on MFS in animal models.

Fbn1-mutant mouse models have been used in numerous studies for several years. The etiology of MFS has been ascribed to dominant-negative effects and haploinsufficiency [24]. The effect of *FBN1* mutations in patients with MFS has been successfully mimicked in mouse models [19]. The utilization of such models carrying specific pathogenic variants has been deemed suitable for the study of early onset and severe symptoms in MFS [25].

Animal models must display all features associated with the disease being studied, including heterogeneity of symptoms, penetrance, and expressivity, to facilitate extrapolation of observations and results to humans. Herein, we discuss the utility and validity of GE mouse and pig MFS models. Additionally, we highlight the difficulties and challenges associated with generating a pig model for an inherited disorder caused by the haploinsufficient mode of gene action.

Characteristics of Mouse MFS Models

Mouse MFS models developed using several background strains, including 129/Sv and C57BL/6, have been used to study the molecular pathogenesis and phenotypic variability of this disease [26]. These models include the introduction of genetic variations that result in *FBN1* loss-of-function mutations in exons 19–24 of mouse *Fbn1* [27]. Genetic variants in mouse models have been found to be comparable to the corresponding human variants, such as the missense change C1039Y [28]. Although they vary between mouse strains and different variants, the main symptoms exhibited by MFS model mice generally include a high incidence of emphysema, deterioration of the aortic wall, kyphosis, aortic aneurysms and dissection, and fragmentation of elastic fibers throughout the vessel wall [29–31]. This section focuses on the variance and associated characteristics of mouse MFS models from two perspectives, namely, those of expressivity and penetrance.

Variable Expressivity of MFS Symptoms within and among Mouse Strains

Expressivity refers to the range of types and severity of symptoms observed in individuals with the same genetic condition. The expression of MFS symptoms in mouse models has been found to result in inter- and intra-strain variations, even in the presence of identical genetic mutations. Previous studies have reported wide clinical variability in mouse MFS models [32]. Lima *et al*. [29] discovered considerable variations in disease severity among individual mice in an MFS mouse model derived from a 129/Sv background. The model appropriately represented the clinical heterogeneity associated with MFS in humans in terms of cardiovascular phenotypes. Furthermore, a positive correlation was observed between age and the incidence of MFS in this model, which exemplified the variability in phenotypic expression within a cohort with the same genetic background. Schwill *et al*. [32] investigated *Fbn1* hypomorphic mice (mgR/mgR) created by the insertion of a neomycin cassette between exons 18 and 19 of *Fbn1*. The mice exhibited extensive variability in symptom onset, including diaphragmatic hernia, kyphosis, scoliosis, and rectal prolapse, thereby highlighting the wide-ranging heterogeneity in the expressivity of symptoms within a single mouse strain.

Different mouse strains exhibit distinct forms of the same trait because of homozygosity acquired through inbreeding. Therefore, mouse MFS models with distinct genetic backgrounds exhibit phenotypic variations despite harboring the same pathogenic mutation. Lima *et al*. [29] compared the phenotypes of mouse models derived from 129/Sv and C57BL/6 (B6) backgrounds, both of which carried the same genetic mutation within *Fbn1*. Their results demonstrated that 129/Sv mice, which were heterozygous for this mutation, displayed a more severe skeletal phenotype than B6 mice. Furthermore, their results revealed variability in the age at symptom onset between the two strains. At 3 months of age, 129/Sv mice exhibited significant thickness of the aortic media, while B6 mice were asymptomatic. However, at 9 months, the thickness of the aortic media was comparable in mice of both the strains.

Variable Penetrance of MFS Symptoms within and among Mouse Strains

Penetrance refers to the proportion of individuals harboring a particular pathogenic mutation who develop symptoms of genetic disorders. MFS is characterized by reduced penetrance among familial individuals carrying pathogenic mutations in *FBN1* [33]. Lima *et al*. [29] reported variable penetrance of identical *Fbn1* mutations between different generations of 129/Sv congenic mice crossed with CD-1 mice, as assessed by the presence of MFS related phenotypes. The F2 offspring demonstrated a more frequent onset of symptoms with greater penetrance than the F1 generation. Phenotypic variability could be ascribed, at least in part, to the genetic heterogeneity of the intercrossed mice.

De Souza *et al*. [34] reported a high penetrance of MFS symptoms in heterozygous *Fbn1* mutant mice with a 129/Sv background. These included descending thoracic aortic aneurysm and/or dissection, aortic dissection with false lumen and tunica intima rupture, and spinal tortuosity. In contrast, heterozygous mutant mice with a mixed 129/Sv/CD-1 background had significantly reduced penetrance of MFS symptoms, except for the sporadic incidence of spinal deformities [29]. Fernandes *et al*. [35] observed high penetrance with wide variation in phenotypic severity in F2 heterozygous *Fbn1* mutant mice of mixed background with 129/Sv and B6, whereas F1 animals of this strain showed little variation and less severe symptoms. The high phenotypic variation in symptoms involving the skeletal, pulmonary, and cardiovascular systems in these mice was more prominent than that in the 129/Sv parental strain. Additionally, high heritability and distribution of these phenotypes were observed between generations of 129 Sv/B6 mice.

The above-mentioned studies indicate variation in the penetrance of MFS symptoms in mouse models with different backgrounds, which explains the observation of a wide clinical spectrum in each MFS mouse model.

Characteristics of Pig MFS Model: Variable Penetrance and Expressivity of the MFS Symptoms in the *FBN1*$^{mut/+}$ Pig Pedigree

We generated *FBN1*$^{mut/+}$ (Glu433AsnfsX98/WT) pigs via somatic cell nuclear transfer (SCNT) under two sets of conditions [11]. The first involved the transfer of SCNT embryos at the blastocyst stage into recipient gilts after long-term culture of 5 days. Among the eight *FBN1*$^{mut/+}$ siblings, four displayed a diverse array of MFS symptoms, including scoliosis, pectus excavatum, delayed mineralization of the epiphysis, and elastic fiber abnormality of the aortic wall, many of which were observed in the neonatal period. The second group of 10 cloned pigs was obtained from SCNT embryos transferred at the early cleavage stage without long-term culture. Symptom onset was observed in only two of these siblings at maturity, without any abnormalities during the neonatal period. The *in vitro*

manipulation of embryos, including culture, epigenetically modifies gene expression and may be responsible for the manifestation of MFS symptoms in cloned siblings of the former group but not of the latter [36, 37].

The expressivity and penetrance of MFS symptoms in *FBN1*$^{mut/+}$ pigs were determined by analyzing the pig pedigree obtained by mating the founder-cloned pig (Generation-0, G0) with wild type (WT) pigs. This was done to verify the effect of epigenetic modifications on cloned pigs obtained by SCNT, since epigenetic modifications that affect the phenotype of cloned animals are reportedly eliminated in the next generation [38]. Observations made up to the 4th generation of cloned *FBN1*$^{mut/+}$ boar descendants are depicted in the pedigree in Fig. 1. A total of 14, including three stillborn G1 *FBN1*$^{mut/+}$offspring in three litters, were obtained by mating unaffected boars (W198 and W226) from G0 founder clones with two WT females. The incidence of MFS symptoms in these animals was low, ranging from 0 (0/3) to 25% (1/4) in *FBN1*$^{mut/+}$ pigs of each litter.

The siblings (Fig. 1: G2-1) obtained by mating an affected G1 male (W217) and an unaffected G1 female (W259) resulted in

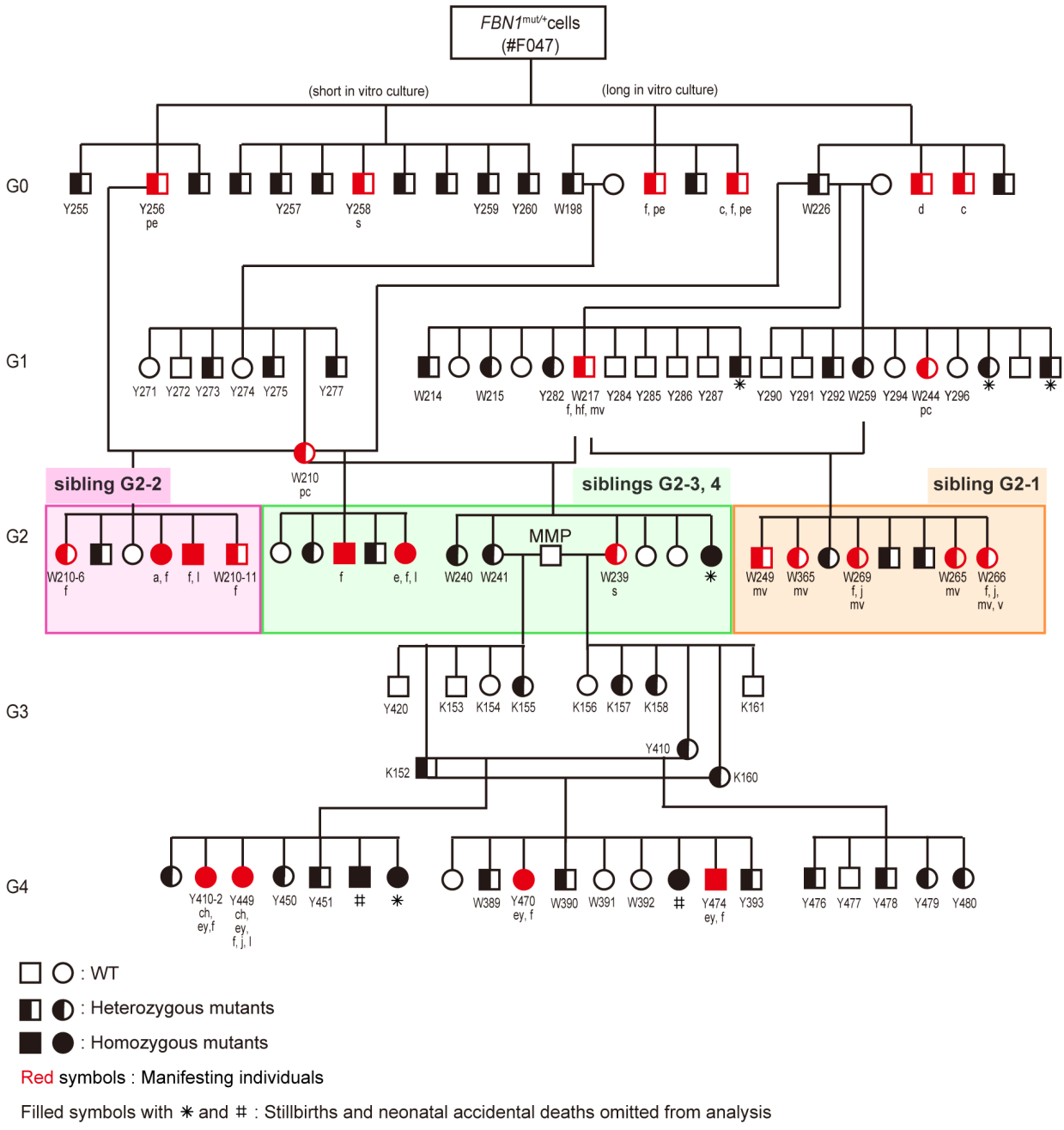

Fig. 1. Manifestation of the MFS symptoms in the *FBN1*$^{mut/+}$ pig pedigree. Founder *FBN1*$^{mut/+}$ cloned pigs (G0; Y256, W198, W226) were generated from nuclear donor cells with genetic background of Large White/Landrace × Duroc. They produced a total of 46 *FBN1*$^{mut/+}$ progeny including 3 stillborn offspring (*) over 4 generations. The WT pigs used for mating with the *FBN1*$^{mut/+}$ pigs were of the same strain as the nuclear donor cells of Microminipig (MMP, Fuji Micra Inc., Japan). Manifestation of the MFS symptoms in the 43 live pigs were analyzed by laparotomy on day 1 to 1215 postpartum. Squares and circles indicate males and females, respectively. a, Aortic dissection; c, cleft palate; ch, cardiac hypertrophy; d, delayed bone mineralization; e, ectopia lentis; ey, eye abnormalities; f, fragmentation of elastic fibers; hf, heart failure; ip, irregular pulse; j, joint hypermobility; l, lipodystrophy; mv, mitral valve thickening; pc, pectus carinatum; pe, pectus excavatum; s, scoliosis; v, expantion of valsalva cave.

as much as 62.5% (5/8) G2 *FBN1*$^{mut/+}$ pigs. Furthermore, a backcross between an affected G1 female (W210) and an affected G0 boar (Y256) resulted in a similarly high incidence of symptoms in 66.7% (2/3) of G2 *FBN1*$^{mut/+}$ progeny (Fig. 1: G2-2). However, the same affected G1 female gave rise to G2 *FBN1*$^{mut/+}$ progeny with a limited manifestation of symptoms (Fig. 1: G2-3, 4) when mated with other boars. *FBN1*$^{mut/+}$ pigs in the G3 and G4 progenies also displayed low penetrance of symptoms. However, the data do not necessarily indicate a change in the nature of the *FBN1* variant across generations because G4 *FBN1*$^{mut/mut}$ progeny developed symptoms.

The analysis of 43 *FBN1*$^{mut/+}$ pigs from G1 to G4 revealed that as many as 81.8% (9/11) of the manifesting animals displayed MFS symptoms 662 days postpartum (Fig. 2). It would be fair to say that the phenotypes of these progeny demonstrate the actual effect of the *FBN1*$^{Glu433AsnfsX98/WT}$ genotype, that is characterized by late onset of MFS. The phenotypic diversity and neonatal onset observed in the G0 cloned founder animals appeared to diminish in pigs after G1, with cardiovascular lesions likely to be the main symptom in G2–G4 individuals (Table 1). While there may be an increase in the expressivity and penetrance of symptoms in the offspring when one or both parents are affected, this could not be conclusively concluded based on the limited data obtained from this study.

Challenges and Prospects in Disease Model Pigs with Haploinsufficiency

The *FBN1*$^{mut/+}$ pedigree faithfully reproduced the variable and late-onset pathogenesis of MFS. Although late-onset pathogenesis may be disadvantageous in certain experimental setups, it allows research to be conducted on the pre-symptomatic stages of the disease. Studies that utilize animal models of diseases with a long pre-symptomatic stage require a guaranteed onset of symptoms at certain stages of disease progression. Predicting and controlling the kinetics of MFS symptom onset in pig models remains a significant challenge for future research.

MFS is caused by haploinsufficiency, and symptom onset is based on the expression levels of *FBN1* encoded by the normal allele [39]. Therefore, the ability to regulate the expression of the normal *FBN1* allele is crucial for improving the utility and practicality of using *FBN1*$^{mut/+}$ pigs as an MFS model. However, identification and regulation of secondary factors or modifiers of *FBN1* expression is a major challenge. We observed fluctuations in the DNA methylation status of the CpG island shore of the *FBN1* promoter [40], which may be exploited to regulate *FBN1* expression. Although techniques such as RNA interference [41] and microRNA [42] may be employed, rigorous investigation is essential to optimize the manifestation of symptoms in *FBN1*$^{mut/+}$ MFS pig models. Recent advances in genetic and reproductive engineering technologies have enabled the development of pigs with monogenic diseases. The *FBN1*$^{mut/+}$ pigs described above exemplify the necessity of research aimed at improving GE pigs as models for diseases caused by haploinsufficiency.

Conflict of interests: There is no conflict of interest.

Acknowledgements

The studies on MFS model pigs described in this paper received financial support from the Meiji University International Institute for Bio-Resource Research, Leading Advanced Project for Medical Innovation, AMED, and Grants-in-Aid for Scientific Research, JSPS (Grant No.21H04755).

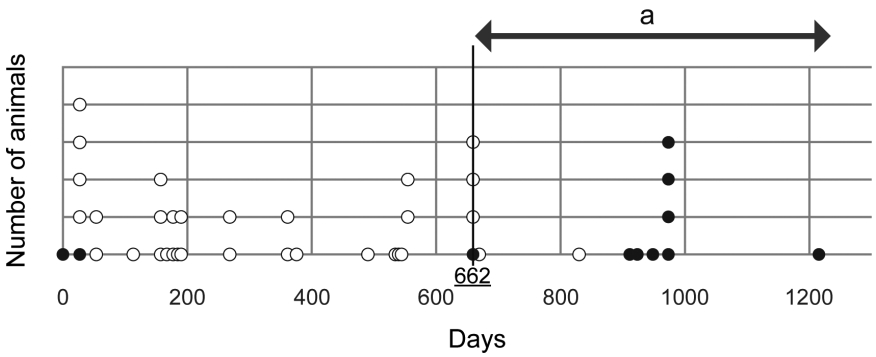

Fig. 2. Variable penetrance of the MFS symptoms in the *FBN1*$^{mut/+}$ pig pedigree. Most (81.8%, 9/11) of the manifesting animals displayed the MFS symptoms after 662 days postpartum (a). Solid circles: pigs manifesting symptoms, blank circles: pigs without manifestation.

Table 1. Symptoms observed in the manifesting *FBN1*$^{mut/+}$ pigs

Pig code	Sex	Age at laparotomy (days)	Symptoms	
			Skeletal	Cardiovascular
W210	F	943	pectus carinatum	
W217	M	1215		mitral valve thickening, cardiac hypertrophy, fragmentation of elastic fibers
W244	F	921	pectus carinatum	
W210-6	F	29		fragmentation of elastic fibers
W210-11	M	1		fragmentation of elastic fibers
W239	F	662	scoliosis	
W249	M	914		mitral valve thickening
W365	F	970		mitral valve thickening
W269	F	970	joint hypermobility	mitral valve thickening, fragmentation of elastic fibers
W265	F	970		mitral valve thickening
W266	F	970	joint hypermobility	mitral valve thickening, expansion of valsalva cave, fragmentation of elastic fibers

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